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13. Abstract PNCK, a novel pregnancy upregulated CaM kinase is upregulated in the mouse mammary gland during pregnancy, particularly in late pregnancy when alveolar epithelial cells exit the cell cycle and differentiate. PNCK is also upregulated in cultured over-confluent and serum-starved cells compared to actively growing mammary epithelial cells, suggesting that PNCK expression is inversely related to proliferation. Interestingly, PNCK is expressed in a subset of human breast tumor samples, but not in adjacent normal breast tissue. In mouse mammary carcinoma cell lines derived from MMTV-c-Myc driven tumors, PNCK expression is prominent, while no association is found between PNCK and MMTV-neu or MMTV-ras tumors. Recently, our lab discovered that a prominent survival pathway incident from the EGFR required calcium and calmodulin for the activation of Akt and the inhibition of c-Myc mediated apoptosis in c-Myc overexpressing mammary carcinoma cells. We hypothesize that PNCK (a calcium and calmodulin dependent protein) opposes the Akt activation properties of CaM in c-Myc overexpressing cells and in mammary carcinoma cells in general. We successfully cloned and expressed the human *PNCK* transiently in epithelial cells. Our aim to functionally characterize PNCK in stable mammary carcinoma cells has been prevented by technical problems. We will now focus on optimizing a transient model of PNCK expression to uncover its function in mammary carcinoma cells.

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INTRODUCTION

Introduction: Breast cancer arises in part because inappropriate growth and survival signals encourage a cell to divide and grow when it should not (1-3). Another component of the cellular transformation process is the inability of a cell to apoptose in the presence of these inappropriate oncogenic signals (1-2). The majority of human breast cancers overexpress c-Myc, leading to genomic instability, inappropriate cell cycle progression and interestingly to apoptosis (4,5). In the past, our laboratory and others have focused on uncovering molecular pathways responsible for mediating c-Myc's apoptotic effect (6,7,8). In the process we have uncovered several pathways that are responsible for inhibiting c-Myc-mediated apoptosis. One of the pathways which provided a strong survival signal in our model (Myc83, mouse mammary carcinoma cells from MMTV-*c-myc* tumor) was the PI3K/Akt pathway (9). Not only did EGF stimulation of Myc83 cells result in the immediate activation of Akt, but also this activation prevented apoptosis of these cells (9). When a constitutively activated Akt molecule was permanently introduced into Myc83 cells, they were dramatically protected from apoptosis induced by a PD153035 tyrosine kinase inhibition-mediated EGFR signaling block (9). In search of upstream regulators of Akt, our lab discovered that EGF-mediated Akt activation was diminished or abolished by calcium chealation and inhibitors of calcium-calmodulin, suggesting that calcium signaling molecules in Myc83 cells were important modulators of EGF, signaling upstream of Akt. Currently, our group is uncovering the exact molecular components responsible for the survival effect of calcium and calmodulin.

Calcium, a known and important intracellular signaling molecule, exerts many of its signaling properties by interaction with a calcium binding-protein called calmodulin (10-12). Calmodulin, coupled with calcium are able to interact with and activate a variety of kinases termed calcium-calmodulin dependent protein kinases (CaM kinases) (10-12). CaM kinases are extensively studied in the nervous system, where they play a critical role in intra-neuronal signaling and in muscular tissue where many of their substrates have been identified including cytoskeletal proteins such as actin and myosin (10-12). Their role in mammary epithelium signaling was virtually unknown until a few years ago.

Recently, however, the role of certain calcium/calmodulin dependent protein kinases has been explored in a number of epithelial tissues and two CaM kinases have been discovered to play a potential tumor suppressive role in cancer. DAP kinase (Death associated protein kinase), in particular, has been extensively studied and well characterized as a pro-apoptotic protein in response to INF-gamma, TNFα and CD 95 ligand (13,14). DAP kinase is lost in 30% of human breast cancers, suggesting it may function as a tumor suppressor in transformed cells (13,14).

In 2000, an intriguing CaM kinase was discovered; it was upregulated in the mammary gland during pregnancy and expressed in human mammary carcinomas. In particular, the CaM kinase was expressed in MMTV-c-Myc transgenic mouse mammary tumor cell lines. This new mouse CaM kinase is called PNCK (pregnancy-upregulated nonubiquitous CaM kinase) (15). This CaM kinase, PNCK, had similarities to CaM kinase I, and was upregulated during mouse mammary glad development and specifically expressed in the adult mouse during pregnancy (15, 16). Interestingly, PNCK was expressed at the highest levels in late pregnancy, when alveolar epithelial cells exit the cell cycle and differentiate (16). PNCK was also upregulated in cultured, over-confluent and serum-starved cells compared to actively growing mammary epithelial cells (16). These findings suggest that PNCK expression is inversely related to proliferation in mammary epithelial cells. Additionally, PNCK was upregulated in a number of human breast

tumor samples, but not in benign tissue. PNCK was also upregulated in a subset of human breast cancer cell lines and, interestingly, specifically upregulated in mouse mammary tumors resulting from MMTV-driven overexpression of c-Myc, but not from tumors derived from other MMTV-oncogene-driven transgenic mice (16). These observations suggest that PNCK is also associated with transformation of the mammary epithelium, and possibly in a c-Myc associated manner.

As mentioned above, we have recently carried out a series of investigations that uncover the survival mechanism(s) employed in MMTV-c-Myc carcinoma cells derived from mouse mammary tumors. In these cells, which overexpress calmodulin, a unique role of calcium and calmodulin was uncovered, as we observed that calmodulin can be activated in an EGF and calcium-dependent manner to mediate PI3 kinase and resultant Akt activity.

Our lab hypothesized that PNCK expression opposes the Akt activating properties of CaM in c-Myc-expressing mouse mammary carcinoma cells (Myc83). The relationship between PNCK and Akt in regulation of EGF signaling, upstream of Akt in mouse mammary carcinoma cells is currently being investigated in our lab.

PNKC is a novel, and completely uncharacterized protein. In general CaM kinases' role in mammary epithelial cell proliferation and transformation are virtually unexplored with the only known exception being DAP kinase, a CaM kinase which has demonstrated a potentially strong tumor-suppressive function (13,14). Because of these facts, and because of the potential therapeutic benefit of characterizing such a novel kinase, we initially proposed to clone the human PNCK gene and investigate its role in human breast cancer cells.

BODY:

APPROVED REVISED STATEMENT OF WORK:

Hypothesis: We propose that PNCK, the pregnancy upregulated nonubiquitous CaM kinase, has an anti-proliferative or pro-apoptotic effect on mammary epithelial cells during or after the transformation process.

Aim 1: We aim to clone the human PNCK gene and functionally characterize its role in mammary carcinoma cells.

Aim 1a: Cloning of the human PNCK gene. [STATUS: Completed]

Aim 1b: Express PNCK in a transient and stable manner, as well as PNCK mutants in human mammary carcinoma cells compared to empty vector control. [STATUS: Ongoing experiments]

Aim 1c: Functionally determine the effect of PNCK expression and PNCK mutants on cell proliferation and apoptosis *in vitro*. [STATUS: Ongoing]

Aim 2: We aim to clone the PNCK promoter and determine the fundamental transcriptional regulatory elements controlling PNCK expression.

Aim 2a: Clone the PNCK promoter. [STATUS: Not yet begun]

Aim 2b: Promoter analysis to determine basic transcriptional regulation of the human PNCK gene. [STATUS: Not yet begun]

Aim 2c: Determine the relationship between c-Myc and PNCK regulation. [STATUS: Not yet begun]

Progress Aim 1:

Hypothesis: We propose that PNCK, the pregnancy upregulated nonubiquitous, CaM kinase has an anti-proliferative or pro-apoptotic effect on mammary epithelial cells during or after the transformation process.

Aim 1: We aim to clone the human PNCK gene and functionally characterize its role in mammary carcinoma cells.

Aim 1a: Cloning of the human PNCK gene.

Since the mouse gene has been cloned, sequenced and mapped to the Xq28 chromosome, we cloned the human PNCK by using the mouse sequence and screening human EST databases for homologous matches. Search of the EST database revealed a recently published nucleotide GeneBank entry U52111, which sequenced through the entire human Xq28 region. Within that entry was a human gene similar to CaM kinase I (17). The DNA sequence of the CaM kinase similar to human CaM kinase I was 89%

homologous to the mouse PNCK gene sequence and 95% conserved at the protein level (17). With this information, we assumed that the gene published in the Xq28 sequence was the human *PNCK* gene. Using the human U52111 sequence, we searched the EST data base for possible image clones and found 2 possible matches from a cDNA male human brain library enriched for full-length clones. The image clones were purchased and sequenced in total. The human PNCK gene was obtained by PCR of those clones (see Figure 1).

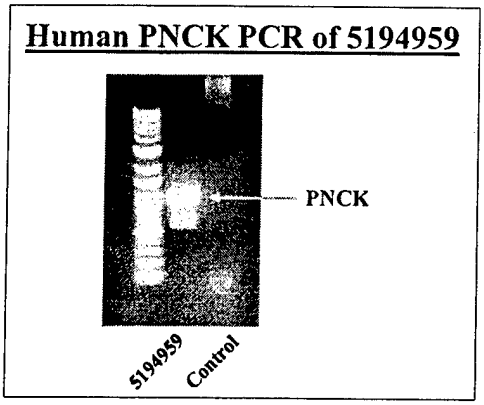

Figure 1: Human PNCK was cloned by PCR of express sequence tag number 5194959. Sequencing revealed that human PNCK is 89% homologous to the mouse PNCK gene and 95% conserved at the protein level.

Next we cloned the PNCK into a phCMV-HA-tagged expression vector (Gene Therapy Systems, Inc., San Diego, CA) and verified both the full sequence and proper orientation.

Aim 1b: Transient and stable expression of PNCK, and PNCK mutants in human mammary carcinoma cells compared to empty vector control.

After cloning PNCK into the CMV driven phCMV-HA tagged vector, we verified its expression in COS-7 cells using a rabbit polyclonal antibody generated and purified by our laboratory (see Fig. 2).

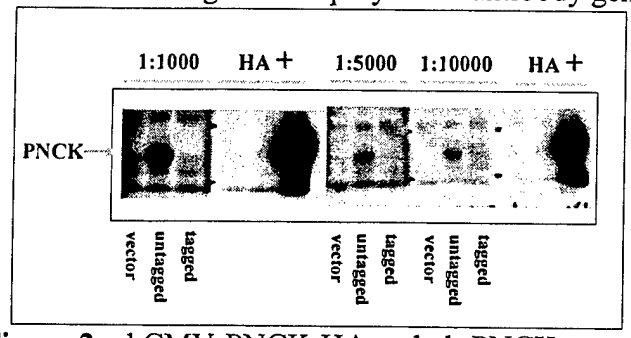

Figure 2. phCMV-PNCK-HA and ph-PNCK expression verified in Cos 7 cells with rabbit polyclonal sepcific PNCK antibody.

Next we generated mammary carcinoma cells that constitutively express PNCK. We chose two cell lines that were negative for the pro-apoptotic kinase DAP-kinase, since DAP kinase might confound our results, MCF-7, T47D. I transiently introduced PNCK into the human breast cancer cell lines by lipid transfection method with LIPOFECTAMINE 2000 (Invitrogen) and selected stable clones *via* exposure to G418 for two to three weeks. Both C-terminal HA-tagged and untagged stable clones were generated in MCF-7 and T47D (Figure 3). The polyclonal antibody developed against the C-terminal portion of PNCK did not detect the HA-tagged PNCK. Commercially available HA antibodies were therefore employed to detect PNCK-HA.

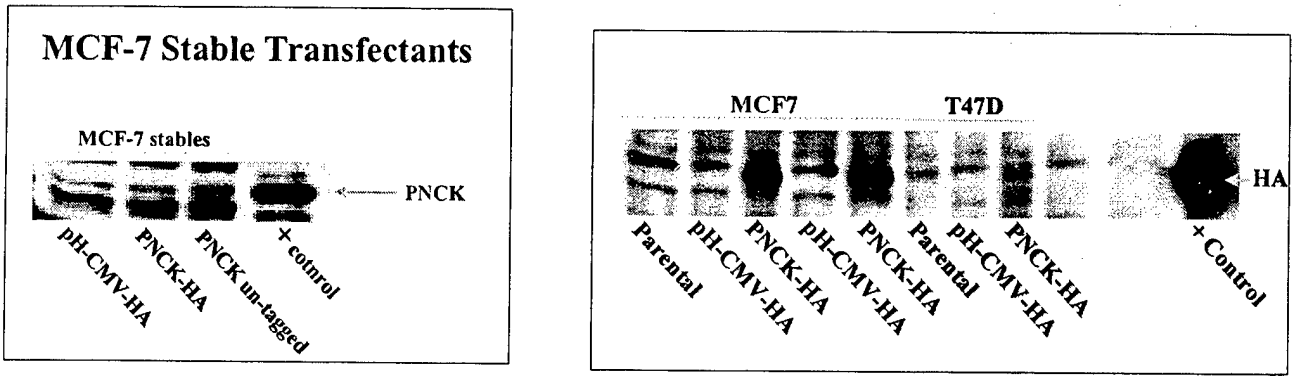

Figure 3. Generation of stable PNCK and PNCK-HA expressing MCF-7 and T47D cells.

After 4 to 6 passages post selection and screening, Pnck expression was lost in MCF-7 and T47D stable cells expressing PNCK (see Figure 4).

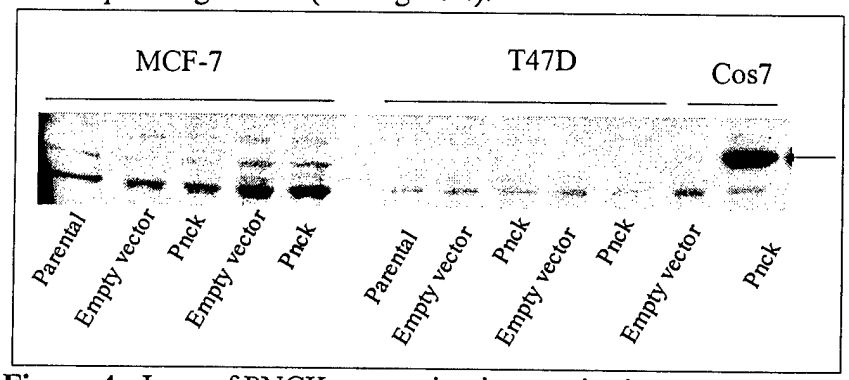

Figure 4: Loss of PNCK expression in constitutive stables after 4-6 passages post selection.

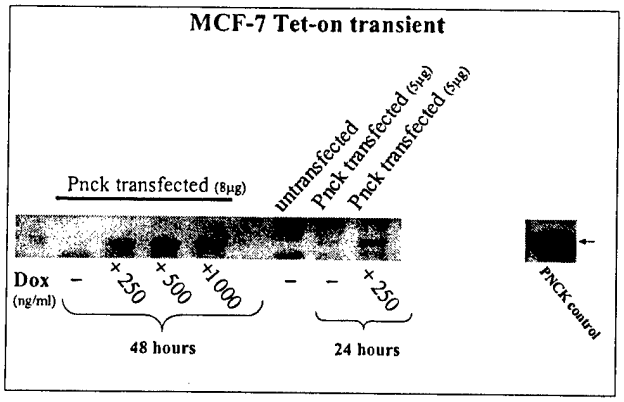

Figure 5: Regulated transient expression of PNCK by doxycycline in MCF-7 Tet-On cells.

Alternative Approach to Aim 1b. Generation of Tetracycline-regulated PNCK-expressingTet-ON MCF-7 cells.

Because constitutive expression of PNCK was not maintained through initial expansion of stable clones, I cloned the *PNCK* gene, both tagged and untagged, into the pUHD 10-3 vector which possesses a tetracycline responsive element (TRE). This was introduced into MCF-7 cells generated to possess the

reverse tetracycline trans-activator (rtTA), obtained from Doug Yee (University of Minnesota). In the presence of tetracycline or Doxycycline, the Tetracycline transactivator is able to activate the tetracycline-responsive element on pUHD 10-3 and drive expression of PNCK. Tetracycline-regulated, MCF-7 Tet-On cells transiently transfected with a TRE driving PNCK (pUHD-10-3-PNCK) demonstrate PNCK expression in response to doxycycline (See Figure 5).

Induction of PNCK by Dox in the stable MCF-7 Tet-On system has proven to be problematic. Specifically, generation of stable inducible clones has proved to be nearly impossible. Twice, Tet-inducible clones were generated, and screened for doxycycline inducibility. After screening over fifty individual clones and ten pooled clones, we have yet to identify a single clone that expresses PNCK upon exposure to DOX. We are now attempting to optimize a transient approach to PNCK expression for functional characterization.

Aim 1c: Functionally determine the effect of expression of PNCK and PNCK mutants on cell proliferation and apoptosis *in vitro.*

Catalytically inactive PNCK mutants and mutants deleting the CaM regulatory domain were generated by site-directed mutagenesis. These will be used in our analysis of PNCK effects on cell cycle and cell death. The mutants generated are listed below:

PNCK MUTANTS:
-Pnck mut/K48M, Pnck mut/K49M and Pnck mut/K48,49M: disruption of ATP binding site (kinase reaction)
-Pnck mut/T171A, direct phosphorylation site mutant
-Pnck mut/W296K, mutation of Calmodulin binding site

Since stable expression of PNCK has proven difficult, we will transiently introduce PNCK into cells. We will begin investigating PNCK's role on cell proliferation and cell death. We will evaluate whether PNCK inhibits cell cycle by transfecting PNCK, PNCK kinase dead mutant and vector control into MCF-7, T47D and MDA-MD 231 and evaluate effects of PNCK on cell function on a cell-by-cell basis. This will be done by selecting for transfected cells by immuno-fluorescence with our Pnck antibody and with the HA antibody. Positive cells will be evaluated for changes in cell cycle. FACS analysis will be performed comparing the percentage of cells in G1, S, G2 and M phases, using PNCK and PNCK mutants *vs.* vector control. The effect on PNCK and PNCK mutants on apoptosis will be determined by Hoechst staining of nuclear morphology, compared to vector controls. Comparison of apoptotic death between PNCK, PNCK mutants and control will be determined by Annexin staining and apoptotic morphology in positive cells. Alternatively, Pnck function may be analyzed on a cell-by-cell basis with co-transfection of Green fluorescent protein, followed by cell sorting for GFP positive cells and then subsequent analysis of cell cycle and apoptotic changes.

If no effect is seen in the PNCK-transfected cells, we will determine if the catalytically inactive mutants can protect breast cancer cells from pro-apoptotic stimuli such as INF-γ, FasL, TNFα, radiation.

To further investigate the mode of action of PNCK in mammary epithelial cells, PNCK may be cloned into a FLAG-tagged vector, expressed in breast cancer cells and its intracellular localization visualized by immunostaining with an anti-FLAG antibody. Changes in cellular location of PNCK, upon exposure to pro-apoptotic stimuli, will also be evaluated with PNCK-FLAG-tagged expression in breast cancer cells.

Aim 2: We aim to clone the PNCK promoter and determine the fundamental transcriptional regulatory elements controlling PNCK expression.

Aim 2a: Clone the PNCK promoter:

Aim 2b: Promoter analysis to determine basic transcriptional regulation of the human PNCK gene.

Aim 2c: Determine the relationship between c-Myc and PNCK regulation.

Progress on Aim 2:
To date, Aim 2 has not been started; we leave aim 2 unchanged.

Aim #2: Year 2: As previously expressed in the revised statement of work:

We aim to clone the PNCK promoter and determine the fundamental transcriptional regulatory elements controlling PNCK expression.

Aim 2a: Clone the PNCK promoter:

In order to understand the basic transcriptional regulation of the human PNCK, we propose cloning of the PNCK promoter. We aim to do this by cloning the PNCK promoter from a human genomic library by using the PCR-based Promoter Finder DNA Walking kit (Promega). Gene specific primers will be derived from the 5'UTR of the human PNCK gene. The full length promoter will cloned by sequenced. The transcription start site of the human gene will be determined using primer extension analysis with nested primers derived from known DNA sequence.

Aim 2b: Promoter analysis to determine basic transcriptional regulation of the human PNCK gene.

First, sequence analysis of the promoter will be performed to determine the presence of consensus transcription factor binding sites present in the human PNCK promoter sequence. To identify the functional promoter elements involved in PNCK gene regulation, progressive 5' deletion mutants will be constructed based on the location of consensus factor binding sites on the promoter. These promoter mutants as well as the wild-type promoter will be cloned into a luciferase vector, transfected into human breast cancer cells and their relative luciferase activity will be assayed. In addition to the 5' deletion mutations, internal mutation and deletions will be made by PCR based site-directed mutagenisis, based on the results of the 5' deletions. These internal deletions and mutations will be cloned into the luciferase vector for transfection into human breast cancer cells.

Aim 2c: Determine the relationship between c-Myc and PNCK regulation.

Since MMTV-*c-myc* but not MMTV-*ras* mice demonstrated a specific upregulation of PNCK in their mammary tumors, c-Myc may be a direct transcriptional regulator of PNCK (16). Using unique, stable c-Myc expressing mammary epithelial cell line created in our laboratory, (MCF10A-Myc) and a 4-OHT regulatable Myc, Myc-ER (MCF10A-MycER) we can determine if c-Myc activity results in PNCK expression and directly investigate the relationship between c-Myc and PNCK regulation (18).

KEY RESEARCH ACCOMPLISHMENTS:

-I have cloned the human *PNCK* gene and expressed it transiently in human mammary carcinoma cells and COS-7 cells.
-I have determined that constitutive stable expression of PNCK is not a reliable way to investigate PNCK function.
-I have generated stable cell lines expressing PNCK and have discovered that PNCK expression is abolished 4-6 passages after selection during expansion of the cell lines.
-I have generated a transient tetracycline responsive system that induces PNCK in response to DOX in MCF-7 cells.
-I have determined that generation of the MCF-7 Tet-ON-PNCK cell line is not realistic.
-We have generated a specific rabbit polyclonal antibody which recognizes PNCK and is used successfully in Western blotting. Additionally, this antibody is being optimized for use in immunofluorescence.

REPORTABLE OUTCOMES:

-Co-authorship of an Experimental Cell Research publication:
Ramljak, D., Coticchia, C., Nishanian, G.T., Saji, M., Ringel, M.D., Conzen, S.D., and Dickson, R.B. Epidermal Growth Factor Inhibition of c-Myc-Mediated Apoptosis through Akt and Erk Involves Bcl-xl Upregulation. Experimental Cell Research 287 (2): 397-410. SEE APPENDIX.

-Co-authorship on a submission to EMBO journal:
Deb, T.B., Coticchia, C.M., Dickson, R.B. Calmodulin mediated PI3 kinase/Akt activation regulates survival of a c-Myc overexpressing mouse mammary carcinoma cell.
Subimitted: EMBO Journal 10-2003.

CONCLUSIONS

The previous stated aims attempt to characterize a novel CaM kinase PNCK both in its function, regulation and activity. The work done cloning and expressing the *PNCK* gene to date has been attempted and in part was successful. Generation of stable expressing PNCK cell lines has proven unsuccessful. Both constitutive expressing PNCK mammary carcinoma cell lines and regulatable, tetracycline-inducible PNCK expressing MCF-7 Tet On cells were not viable. Our focus to date is to optimize a transient method of PNCK expression to employ in our experiments determining PNCK function in mammary epithelial cells. A number of new reagents, developed for PNCK, will aid us in our investigation of PNCK function. These include a specific polyclonal antibody against PNCK used for both western blotting and for immunofluorescence as well as a number of expression vectors driving wild type and PNCK mutants. The next phase of experiments will be performed without anticipated difficulty, as the techniques employed are those that have been utilized successfully in this lab and by this investigator.

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APPENDIX:

1.) D. Ramljak, C.M. Coticchia, T.G. Nishanian, M. Saji, M.D. Ringel, S.D. Conzen, and R.B. Dickson (2003). Epidermal growth factor inhibition of c-Myc-mediated apoptosis through Akt and Erk involves Bcl-Xl upregulation in mammary epithelial cells. Experimental Cell Research, 287: 397-410

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Epidermal growth factor inhibition of c-Myc-mediated apoptosis through Akt and Erk involves Bcl-x_L upregulation in mammary epithelial cells

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Abstract

In earlier studies, we and others have established that activation of EGFR can promote survival in association with upregulation of Bcl-x_L. However, the mechanism responsible for upregulation of Bcl-x_L is unknown. For the current studies we have chosen pro-apoptotic, c-Myc-overexpressing murine mammary epithelial cells (MMECs) derived from MMTV-c-Myc transgenic mouse tumors. We now demonstrate that EGFR activation promotes survival through Akt and Erk1/2. Blockade of EGFR kinase activity and the PI3-K/Akt and MEK/Erk pathways with pharmacological inhibitors resulted in a significant induction of cellular apoptosis, paralleled by a downregulation of both Akt and Erk1/2 proteins. Consistent with a survival-promoting role of Akt, we observed that constitutively activated Akt (Myr-Akt) inhibited apoptosis of pro-apoptotic, c-Myc-overexpressing cells following the inhibition of EGFR tyrosine kinase activity. In addressing possible downstream effectors of EGFR through activated Akt, we detected significant upregulation of Bcl-x_L protein, suggesting this pro-survival protein is a target of Akt in MMECs. By using pharmacological inhibitors of PI3-K/Akt and MEK/Erk together with dominant-negative Akt and Erk1 we observed the decrease in Bcl-x_L protein. Our findings may be of importance for understanding the emerging role of Bcl-x_L as a potential marker of poor prognosis in breast cancer.
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Keywords: Akt; Erk; Bcl-x_L; Epidermal growth factor receptor; c-Myc

Introduction

The c-*myc* gene is thought to play an important role in the onset and progression of breast cancer, where it is commonly amplified and/or overexpressed [1,2]. Depending on the availability of survival factors, cells that constitutively express c-Myc undergo proliferation, growth arrest, or apoptosis through poorly defined mechanisms. Previously, we demonstrated that an epidermal growth factor receptor (EGFR)-mediated survival signaling pathway(s) inhibited apoptosis in c-Myc-induced transgenic mouse mammary tumors [3]. A comparison of tumors and cell lines derived from bitransgenic *tgf a/c-myc* mice with those from single transgenic c-*myc* mice indicated that only the latter model contained a significant fraction of apoptotic cells [3], suggesting that transforming growth factor α (TGFα) protects c-Myc-overexpressing cells from apoptosis in vivo. Further in vitro studies of c-Myc-overexpressing mammary tumor-derived cells confirmed that EGFR ligands, acting through EGFR tyrosine kinase activity, suppressed apoptosis and upregulated the survival molecule Bcl-x_L, at both mRNA and protein levels [4]. However, it was not clear which pathways downstream of EGFR are responsible for these effects.

Presently, there is limited information on the signaling pathways linking EGFR to the regulation of cellular sur-

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vival in mouse and human mammary epithelial and carcinoma cells. However, studies in some nonmammary epithelial cells (hepatic carcinoma cells and keratinocytes) have identified two survival pathways downstream of EGFR: phosphatidylinositol 3-kinase (PI3-K)/Akt and extracellular signal-regulated kinase (Erk1/2). In most cases, the PI3-K/Akt pathway delivers the most potent survival signal downstream of EGFR [5,6].

Akt is a serine–threonine kinase, downstream of PI3-K, which delivers strong survival signals in many cell types [7–9]. Both growth factors and integrins activate Akt through activation of PDK1 and putative PDK2 kinase that subsequently phosphorylate Akt at Thr308 and Ser473 respectively [10]. There are several isoforms of Akt (Akt1, Akt2, Akt3); each has been shown to be expressed at different levels in various tissues [11]. The targets of Akt in epithelial cells, including mouse and human breast cells, include proteins involved in cell growth, metabolism, and apoptosis. The Akt targets involved in apoptosis include Bad, a pro-apoptotic member of the Bcl-2 family of proteins [12], caspase 9 [13], and the forkhead transcription factor [14]. Recently, Akt also has been reported to upregulate the expression of anti-apoptotic proteins in lymphoid cells such as Bcl-x_L [15], Bcl-2 [16], and Mcl-1 [17]. Akt activates NF-κB in both fibroblasts and epithelial cells [18,19].

In most cell types, both growth factors and integrins are capable of activating the MAPK/Erk pathway. Of particular relevance to our studies, the MAPK/Erk pathway was previously shown to convey survival signals in response to EGF [20,21]. Recently, it has been shown that the PI3-K/Akt and the MAPK/Erk pathways can cooperate in the inhibition of Bad in some cell types [22]. However, the pro-survival targets and the interactions of EGFR-activated PI3K/Akt and MAPK/Erk pathways have not been established in murine mammary epithelial cells (MMECs), human breast epithelial cells, or human carcinomas. Our preliminary data indicated that Bad is expressed in MMECs; however, the phosphorylation status of endogenous Bad was difficult to determine due to the lack of reliable antibodies. With the emerging role of activated EGFR in breast cancer, we believed it would be important to determine the anti-apoptotic targets of EGFR-stimulated Akt and Erk, in MMECs and human breast cancer cells.

In our investigations to determine which survival molecules downstream of the EGFR are responsible for upregulation of Bcl-x_L and inhibition of c-Myc-mediated apoptosis in MMECs, we show that constitutively activated Akt provides protection from c-Myc-mediated apoptosis in association with upregulation of the Bcl-x_L protein. By using pharmacological inhibitors of both PI3-K/Akt and MEK/Erk1/2, and dominant-negative (DN)-Erk1 and DN-Akt we detected significant decreases in Bcl-x_L protein expression.

In conclusion, our results demonstrate that EGFR-dependent Akt activity provides a major survival signal against c-Myc-mediated apoptosis in MMECs; both Akt and Erk are obligatory for regulation of Bcl-x_L expression in this model. To our knowledge, these studies provide the first comprehensive evaluation of the role of EGFR-dependent survival molecules in inhibition of c-Myc-mediated apoptosis in murine models of breast cancer.

Experimental procedures

Cell culture and viral infection

Myc83 cells (derived from an MMTV-c-*myc* transgenic mouse mammary tumor in our laboratory) and Comma D cells (immortalized mouse mammary epithelial cells obtained from D. Medina, Baylor College of Medicine) [23] were maintained in a humidified 5% CO_2 environment, in complete medium containing: IMEM (Gibco-BRL, Gaithersburg, MD, USA), 2.5% fetal calf serum (FCS), 10 ng/ml EGF (Upstate Biotechnology Incorporated (UBI), Lake Placid, NY, USA), and 5 μg/ml insulin (Biofluids, Rockville, MD, USA). Myc83 cells were selected as a model because of their high propensity to apoptose after removal of EGF. Comma D cells (with normal c-Myc levels and mutated p53) were used to compare the potency of EGF in activating both Akt and Erk1/2 in MMECs with different c-Myc levels. Retroviruses for Myr-Akt (obtained from N. Hay, University of Illinois) [24] were made by transient transfection of retroviral pBabePuro-Myr-Akt, using Effectene Transfection Reagent (Qiagen, Valencia, CA, USA) into amphotrophic Phoenix cells (a gift of Dr. Gary Nolan, Stanford University, Palo Alto, CA, USA). Viral supernatants were collected and filter-purified. Myc83 cells were infected with pBabePuro vector only or pBabe Myr-Akt in the presence of 4 μg/ml polybrene, and infected stable clones were selected with puromycine (Sigma, St. Louis, MO, USA). Clones were pooled for further analysis. All retrovirally transduced cells were grown in complete medium, with or without addition of EGF. When testing sensitivity to apoptosis, cells were grown in IMEM only, without EGF, FCS, or insulin, or in IMEM with only EGF.

cDNA constructs and transfections

A plasmid containing dominant-negative Akt cDNA (DN-Akt in PCIS2 vector) was kindly provided by Dr. M. Kohn (NIH, Bethesda, MD, USA). The DN-Akt mutant was generated by replacing Thr308 and Ser473 with alanine. Constructs containing dominant-negative Erk1 (K71R) and empty vector pCEP4 were provided by Dr. M. Cobb (University of Texas, Southwestern Medical Center).

Transfections were performed using FuGene 6 (Roche Molecular Biochemicals). Briefly, cells were seeded in 10-cm^2 plates in complete IMEM medium and allowed to

attach overnight. Transfections were performed on 60–80% confluent cells the next day after seeding according to the manufacturer's instructions (Roche). Cell were grown 48 h posttransfections and samples were collected for immunoblotting.

Antibodies and reagents

Rabbit polyclonal anti-total Akt phospho-specific antibodies-recognizing Ser473 and Thr308; anti-Erk1 and Erk2 and anti-Erk1/Erk2 phospho-specific antibodies; Bad and phospho-Bad (Ser 112 and Ser 136) were from Cell Signaling Biolabs (Beverly, MA, USA). Anti-Akt1, anti-Akt2, and Akt3 were from Upstate Biotechnology (UBI), mouse monoclonal anti-α-tubulin and anti-Bcl-2 were from Neomarkers (Fremont, CA, USA); anti-Bcl-x_L (H-62), anti-14-3-3 (C-16), rabbit polyclonal poly (ADP-ribose) polymerase (PARP) (H-250), anti-GSK-3β(0011-A), anti-p85 PI3-K (Z-8), and anti-Raf-1(C-12) were from Santa Cruz (Santa Cruz, CA, USA). The ECL detection reagent was from Amersham (Arlington Heights, IL); PI3-K inhibitor LY 294002 (Biomol Research Laboratories, Plymouth Meeting, PA, USA) and EGFR inhibitor PD153035 (Calbiochem, San Diego, CA, USA); and MAPK kinase (MEK) inhibitor U0126 was from Cell Signaling Biolabs.

EGF stimulation and treatments with inhibitors

Myc83 and Comma D cells (1–2 $\times$ 10^6/ml) were grown in 10-cm^2 culture dishes in complete IMEM medium containing 2.5% FCS, 10 ng/ml EGF (UBI), and 5 μg/ml insulin (Biofluids) until cells reached 60–70% confluence. Complete medium was removed, cells were washed with IX PBS to remove serum and growth factors, and synchronized by serum starvation (grown in IMEM medium with 0.1% fetal bovine serum) for 24 h. After two washes with IX PBS, the cells were stimulated with IMEM medium containing 10 ng/ml EGF for 1, 2.5, 5, 10, and 20 min, washed twice with IX PBS, and lysed in buffer (Cell Signaling Biolabs) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) for protein analysis. Insoluble material was removed by centrifugation. Protein concentrations were determined by a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) and samples were stored at −80°C until used for immunoblotting and in vitro kinase assays.

To test the requirement of active EGFR for EGF-induced Akt and Erk1/Erk2 activation, the synchronized Myc83 cells (as described above) were pretreated with 1 μM PD153035 EGFR tyrosine kinase inhibitor for 3 h, followed by a treatment with 10 ng/ml EGF at 2.5, 5, and 10 min, as described above. The concentration of PD153035 was selected based on literature and pilot studies in our laboratory (data not shown). The control Myc83 cells (−PD153035) were pretreated with an equal volume (10 μL) of DMSO and later stimulated with 10 ng/ml EGF for 2.5 and 5 min. Comma D cells were pretreated with the same concentration of PD153035 (1 μM) and for the same time (3 h) as Myc83 cells, and they were similarly stimulated with 10 ng/ml EGF for 1, 2.5, and 5 min. Control Comma D cells (−PD153035) were treated with EGF for 1, 2.5, 5, 10, and 20 min. Proteins were analyzed for the activity of both Akt and Erk1/Erk2 by immunoblotting, as described in the Western Blotting section of Experimental Procedures.

Induction of apoptosis by prolonged treatment with inhibitors of P13-K, MEK, and EGFR

Myc83pBabePuro and Myc83-Myr-Akt cells were grown in complete IMEM medium to 70–80% confluence in 10-cm^2 plates, and then the complete medium was replaced with IMEM medium containing the following: 10 ng/ml EGF (without serum and insulin); 50 μM LY294002 (Biomol) with and without the EGF; 10 μM U0126 (Cell Signaling Biolabs) with and without EGF, 1 μM PD153035 (Biomol) with and without the EGF. Cells were usually grown for 48 h, but in some cases cells were harvested after only 24 h. In one set of plates, cells were lysed for protein analysis, as described below, while another set of cells, treated equally, was used for evaluation of apoptosis by Hoechst staining.

To test if EGF rescues the Myc83 cells from apoptosis, we performed a separate set experiments in which cells were grown in complete IMEM medium to 70–80% confluence in six-well plates, and then complete medium was replaced with IMEM medium containing EGF ranging from 10 to 60 ng/ml (without serum and insulin). Cells were grown in the presence of 1 μM PD153035 (Biomol) with and without the EGF and, after 48 h were evaluated by Hoechst staining.

Measurement of apoptosis

Apoptosis was evaluated by immunoblotting of PARP protein cleavage, as described in the Western Blotting section of Experimental Procedures, and by Hoechst staining. Briefly, Hoechst staining was performed as follows: after 48 h of treatment with LY294002, U1026, and PD153035, all adherent and floating cells were collected. Samples were centrifuged for 8 min at 1000g at 4°C. Supernatants were discarded, and cell pellets were resuspended in IX PBS containing formaldehyde and Nonidet P-40 (NP-40) and stained with 10 μg/ml Hoechst 33258 dye for apoptotic analysis. At least 500 cells per treatment group were counted with a hemocytometer and evaluated for the presence of condensed nuclei and overall apoptotic appearance.

Akt kinase assay

Protein lysates from control and EGF-stimulated Myc83 and Comma D cells (0, 1, 2.5, 5, 10, and 20 min) were lysed in the buffer described above. Akt kinase activity was analyzed using an Akt Kinase Assay Kit (NEB), which employs GSK-3 as a substrate, according to the manufacturer's instructions. In short, 300 μg of cellular protein was immunoprecipitated with a total Akt antibody immobilized to agarose beads (NEB) at 4°C overnight. Immunoprecipitated Akt protein on beads was washed twice with 1 ml of lysis buffer (described above) and once with kinase buffer (25 mM Tris, pH 7.5; 5-mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3 Vo_4, 10 $MgCl_2$). The beads were then resuspended in 40:1 kinase buffer, containing the Akt protein substrate (1 μg of GSK-3 α/β fusion protein), supplemented with 200 μM of ATP. The assay was carried out according to the manufacturer's instructions (NEB), and protein samples were loaded on 12% SDS–polyacrylamide gels (Novex) and Western immunoblotting was performed, as described below. The membranes were probed with a phospho-specific antibody recognizing GSK-3α-P (at 1:1000 dilution) when phosphorylated by Akt on serine 21 (NEB). The secondary antibody used at 1:2000 was a rabbit polyclonal (NEB). Blots were developed using ECL reagents (Amersham) and exposed to ECL film (Amersham). The intensity of the bands was quantified using a Chemilmager 5500 (Alpha Innotech Corp., San Leandro, CA, USA).

Western blotting

Cell lysates containing a total of 10–15 μg of protein were used for the Western blot analysis. A PhosphoPlus MAPK antibody kit was used according to the manufacturer's recommendations (Cell Signaling Biolabs), to determine Erk activation by immunoblotting in all experimental and control cells. Cellular extracts were analyzed with antibodies against total Erk1/2 (p44 and p42) and with phospho-specific antibodies against phospho-p44/42 (Thr202/Tyr204) (Cell Signaling Biolabs). In addition to Akt kinase assays (see above) the total activity of Akt was determined using phospho-specific antibodies recognizing Akt specifically phosphorylated at Ser^{473} or Thr^{308} (Cell Signaling Biolabs). Akt protein expression was determined by using an antibody recognizing total Akt independent of phosphorylation status (Cell Signaling Biolabs).

All samples for Akt, Erk1/2, p85 PI3-K, Raf-1, and PARP were analyzed on 8% SDS–polyacrylamide gels (Invitrogen, Carlsbad, CA, USA). Those probed for GSK-3β used 10% SDS–polyacrylamide gels, while samples analyzed for Bcl-2, 14-3-3, and Bcl-x_L were tested on 12% SDS–polyacrylamide gels (Novex). Proteins were transferred to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA, USA) by electroblotting. After transfer, membranes were stained with Ponceau protein stain (Sigma) to test for equal loading. The membranes were then washed with IX phosphate-buffered saline /0.1% Tween 20 (PBST) 3 × 10 min. After washing, membranes were blocked with 2% bovine serum albumin (BSA) in for 1 h. After incubation in primary antibody overnight at 4°C, all blots were washed for 3 × 10 min with PBST and probed for 1 h with the corresponding secondary antibody (anti-mouse or anti-rabbit labeled with horseradish peroxidase; from Amersham, Arlington Heights, IL, USA, or from NEB). After three repeated washes in PBST the blots were developed using a chemiluminescence ECL kit (Amersham) and exposed to X-ray film (Amersham). On several occasions, to test equality of loading, membranes were stripped and reprobed with an antibody recognizing α-tubulin, a 57-kDa protein (Neomarkers). The results of immunoblotting were quantified by densitometry using Chemilmager (Alpha Innotech Corp., San Leandro, CA, USA) and the significance of differences in band intensity was evaluated with a Student's t test.

Results

EGF is a potent activator of Akt in mouse mammary epithelial cells

Our previous studies showed that activation of the EGFR by either EGF or TGFα delivers a potent survival signal to mouse mammary epithelial cells overexpressing c-Myc, both in vivo and in vitro [3]. However, it was not clear which survival pathways downstream of the EGFR were responsible for the inhibition of apoptosis. In the present work, we show that in the absence of serum, EGF stimulation of both Myc83 (MMECs derived from mouse transgenic for c-Myc) and Comma D (immortalized MMECs) significantly activates Akt, a potent survival molecule in fibroblasts and several other epithelial cell types [8]. Akt is activated by 10 ng/ml EGF, within 1 min, (2-fold increase), in both Myc83 (Fig. 1A) and Comma D cells (Fig. 1C). Maximum Akt activation in Myc 83 cells (4-fold increase) was seen at 5 min, followed by a decrease to basal activity by 10 min (Fig. 1A). Similar results were obtained for Comma D cells (Fig. 1D). EGF activation of Akt resulted in the phosphorylation of Ser473 (Figs. 1A and D) and Thr308 (data not shown). Phosphorylation of both sites is required for full activation of Akt [9]. As expected, the EGF treatment did not affect the expression levels of total Akt protein in either cell line, as shown by immunoblotting using anti-Akt antibody recognizing all three Akt isoforms (Figs. 1B and E). The activity of Akt, following EGF stimulation, was confirmed by an Akt kinase assay, using GSK-3α fusion protein as a substrate (Figs. 1C and F). Akt has been shown to phosphorylate GSK-3α at serine 21 [25]. By using an antibody that specifically recognizes

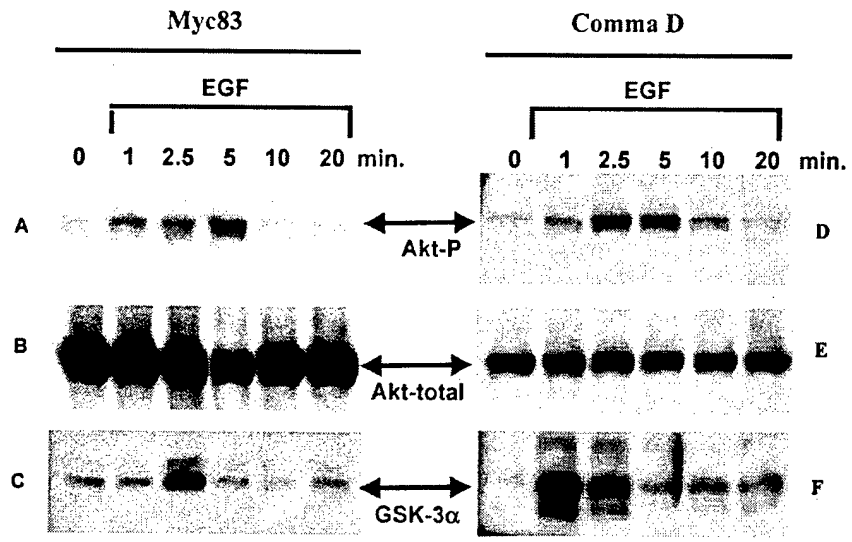


Fig. 1. EGF is a strong activator of Akt in MMECs. (A) Myc83 cells were synchronized by serum starvation (in 0.1% FCS) for 24 h and stimulated with 10 ng/ml EGF in the absence of serum for 1, 2.5, 5, 10, and 20 min. The cells were lysed, and activation of Akt was assayed by immunoblotting, using a phospho-specific antibody recognizing Akt when phosphorylated (activated) at Ser 473. (B) Phospho-Akt blots were stripped and reprobed with an Akt antibody that recognizes Akt independent of its phosphorylation status. (C) In addition, the phosphorylation status of Akt was analyzed by an Akt kinase assay using GSK-3α as a substrate in vitro (D, E). (D–F) Similarly, the effects of EGF on Akt activation and expression also were evaluated in non-c-Myc-overexpressing Comma D cells by immunoblotting and kinase assay. These results are representative of three identical, independent assays using the same lysates and two identical assays using different lysates.

phospho-GSK-3α at serine 21, we confirmed the phosphorylation of GSK-3, a known target of activated Akt, in both Myc83 (Fig. 1C) and Comma D (Fig. 1F). Interestingly, in Comma D cells the maximal activation of GSK-3α at serine 21, as detected by Akt kinase assay, preceded the activation of Akt as detected by immunoblotting. The mechanism responsible is not clear, but it is possible that activation of some other EGFR-activated kinase might precede the activation of Akt and cause phosphorylation of GSK-3α in Comma D cells.

To determine which of the three major Akt isoforms (Akt1, Akt3, Akt3) are expressed and activated after EGF treatment in MMECs, we evaluated their expression levels in Myc83 and Comma D by using antibodies specifically recognizing each isoform (UBI). We detected significant expression of Akt1 and Akt2 in all MMECs analyzed (data not shown), while expression of Akt3 could not be detected in any of the cells with the antibody used in this study (data not shown).

Activation of Erk1/Erk2 by EGF parallels Akt activation

Although in the majority of cell lines tested to date, the PI3-K/Akt pathway has been shown to deliver a stronger

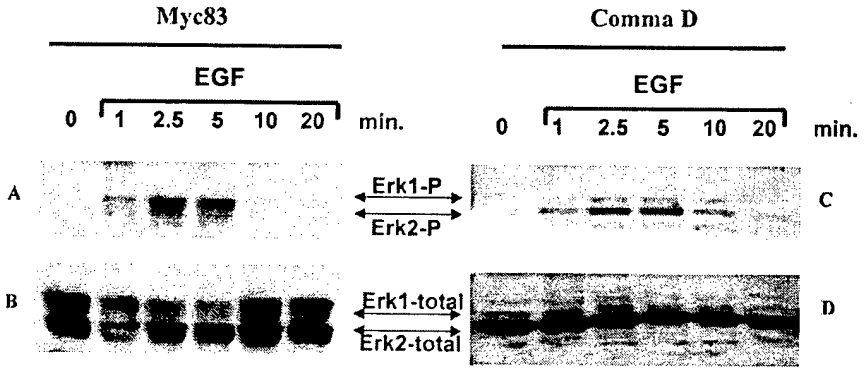


Fig. 2. EGF is a potent activator of Erk1/2 in MMECs. EGF at 10 ng/ml strongly activated Erk1/2 within a time frame similar to that shown for Akt, in both Myc83 (A) and Comma D (C) cells. Phosphorylation status of Erk1/2 was determined by immunoblotting using antibody (NEB) recognizing phospho-Erk1/2. Both blots for phospho-Erk were stripped and reprobed with antibody recognizing Erk1/2 independent of phosphorylation status in both Myc83 (B) and Comma D (D) cells.

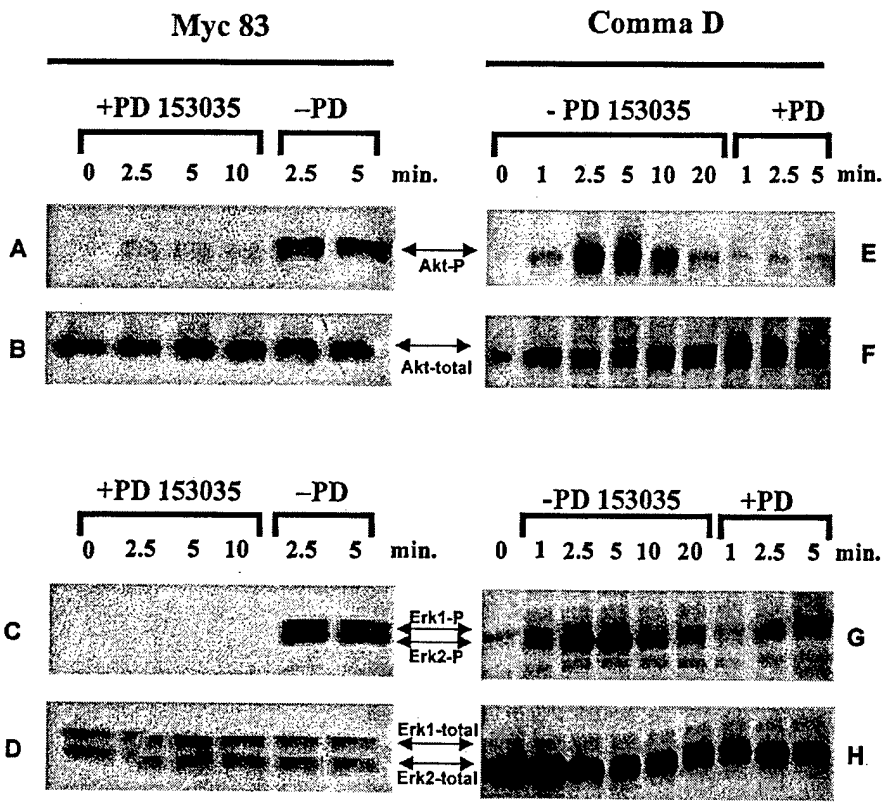


Fig. 3. Effect of a pharmacological inhibitor of EGFR kinase activity on activation and expression of both Akt and Erk1/2 in MMECs. The EGFR tyrosine kinase inhibitor PD153035 (1 μM) completely blocked activity of Akt in Myc83 (A) and Comma D (E) cells. The blots were reprobed with an antibody recognizing total Akt to demonstrate that while PD153035 inhibits the activation of Akt, it has no effect on Akt expression in Myc83 (B) and in Comma D (F) cells. While treatment with PD153035 completely blocked the activation of Erk1/2 in Myc83 cells (C), it did not affect the activation of Erk1/2 in Comma D cells (G). Blots for phospho Erk1/2 were reprobed with antibody recognizing total Erk1/2. Expression of Erk1/2 was unchanged by PD153035 treatment in both Myc83 (D) and Comma D (H) cells. These results are representative of two independent assays.

survival signal compared to the MEK/Erk pathway, in some epithelial cells, both pathways appear to be equally important in delivering survival signals [26]. Therefore, we next determined the status of Erk1/Erk2. The results show that EGF is capable of specifically activating Erk1/Erk2 in both cell lines (Figs. 2A and C), within a similar time frame as shown for Akt (Figs. 1A and D). In each cell line, the highest Erk1/Erk2 activity was detected at 2.5 and 5 min (2.5-fold); similar to Akt, the activity declined by 10 min. In Myc83 cells, the signal for Erk1 was the stronger (3-fold increase) of the two isoforms (Fig. 2A), while in Comma D cells, Erk2 was predominantly activated (2.5-fold) (Fig. 2C). The expression of total protein levels for Erk1 and Erk2 (Figs. 2B and D) was mostly unchanged by EGF treatment in both cells, with the exception of samples with phosporylated Erk1/2 in Myc83 cells, in which we detected less of total Erk1/2. Although the mechanism responsible is not clear, it is possible that the variation in Erk immunoreactivity, shown in Fig. 2, may simply reflect differential reactivity of the phosphorylated species of these kinases, rather than regulation of their levels.

Inhibition of EGFR tyrosine kinase prevents activation of both Akt and Erk1/Erk2 in c-Myc-overexpressing MMECs

To test the requirement of activated EGFR kinase in the activation of Akt, Erk1, and Erk2, we blocked EGFR tyrosine kinase with PD153035, followed by EGF treatment. In both Myc83 and Comma D cells, inhibition of EGFR kinase activity completely abolished EGFR-mediated activation of Akt (Figs. 3A and E), without affecting total protein levels (Figs. 3B and F). As expected, EGF treatment strongly activated Akt in cells treated with vehicle (DMSO) (Figs. 3A and E). However, only in Myc83 cells was blockade of the EGFR tyrosine kinase activity capable of inhibiting activation of Erk1/Erk2 (Fig. 3C), without affecting the expression levels of Erk1/Erk2 proteins (Fig. 3D). Interestingly, inhibiting EGFR with PD153035 in Comma D cells did not prevent activation of Erk1/Erk2 by EGF (Fig. 3G). Regardless of the presence or absence of PD153035, both Erk1 and Erk2 were strongly activated within 1 min, with increasing activity up to 5 min; however, the expression of neither Erk1 nor Erk2 was affected (Fig. 3H).

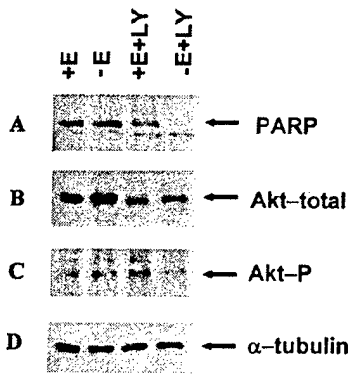


Fig. 4. Prolonged inhibition of PI3-K activity in Myc83 cells causes a decrease in Akt protein. Myc83 cells were grown in the presence or absence of EGF (+EGF, −EGF, respectively), and were treated with 50 μM LY294002 or DMSO for 48 h. (A) Apoptosis was evaluated by immunoblotting of PARP cleavage. Also, the same lysates were analyzed for the expression of Akt protein (B) and the phosphorylation status of Akt (C) by using a phospho-specific Akt (Ser473) antibody. To evaluate if the apoptotic process affects other proteins besides Akt, expression of α-tubulin (a loading control) (D) was evaluated. The results are representative of two independent assays.

Prolonged inhibition of PI3-K activity leads to apoptosis and a decrease in protein levels of both Akt and Erk1/Erk2

Recently, some evidence in the literature suggests that the Akt protein is cleaved by caspases late in the apoptotic process induced by UV irradiation, Fas ligation, and etoposide in human Jurkat cells [27]. Therefore we tested the status of the Akt protein in apoptotic Myc83 cells, following the prolonged inhibition (48 h) of PI3-K activity with 50 μM LY294002. In these experiments, we compared levels of Akt protein in LY294002-treated and untreated Myc83 cells, grown in the presence and absence of EGF. First, by evaluating cleavage of the full-length form of PARP (an early indicator of apoptosis) we found that prolonged treatment with LY294002 leads to apoptosis of Myc83 cells, regardless of the presence or absence of EGF (Fig. 4A). In the same protein lysates, apoptosis was paralleled by a prominent decrease in Akt levels (2.5-fold), independent of the presence of EGF in the growth medium (Fig. 4B). The decrease in Akt phosphorylation was detected in the sample treated with LY294002 in the absence of EGF (Fig. 4C). Furthermore, in the same samples where a decrease in the Akt protein expression and phosphorylation was detected, expression of α-tubulin protein (loading control) was unchanged (Fig. 4D), suggesting that the prolonged inhibition of the PI3-K in c-Myc-overexpressing cells specifically caused downregulation and proteolysis of the Akt protein.

To evaluate how early in the apoptotic process this downregulation of Akt was occurring, we analyzed protein lysates of Myc83 cells treated with the same concentration of LY294002 (50 μM), with and without EGF, for periods of 24 and 48 h. Again, PARP cleavage immunoblotting was used to evaluate apoptosis (Fig. 5A), and the same protein samples were further evaluated for the expression of Akt. Interestingly, as early as 24 h after treatment with LY294002, expression of Akt protein decreased (Fig. 5B), preceding the onset of apoptosis as detected by PARP cleavage (Fig. 5A). However, by 48 h, when a large percentage of the cells underwent apoptosis, as determined by PARP cleavage (Fig. 5A), the decrease in Akt protein level was more prominent (3-fold) (Fig. 5B). The mechanism responsible for decreased steady-state levels of Akt is currently under investigation.

To determine if the prolonged inhibition of PI3-K over 24 and 48 hours specifically is causing Akt downregulation, we evaluated the status of Erk1 and Erk2, Raf-1 (previously shown, together with Akt, to be cleaved late in the apoptotic process in Jurkat cells) [27], and p85 PI3-K. Similar to Akt, the expression of both Erk1 and 2 proteins decreased in the same protein lysates, in which we detected the downregulation of the Akt, following treatment with LY294002 (Fig. 5C). The decrease in Erk1 was more pronounced (2.5-fold), in comparison to Erk2 (Fig. 5C). In contrast to the reported decrease in expression of Raf-1 protein in apoptotic Jurkat cells [27], we did not detect any change in expression of Raf-1 (Fig. 5D) in apoptotic Myc83 cells. However, expression of p85 PI3-K was slightly decreased 48 h after EGF removal, and in LY294002-treated Myc83 cells, in the ab-

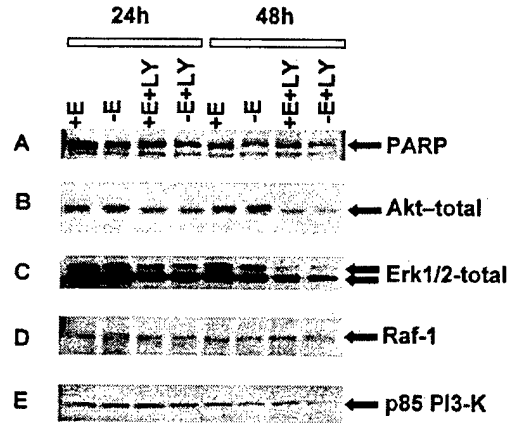


Fig. 5. Inhibition of PI3-K activity caused a decrease in expression of both Akt and Erk1/2 proteins 24 h before the onset of apoptosis. To test how early the inhibition of PI3-K activity causes the decrease in Akt expression, samples of Myc83 cells were treated with EGF (+EGF), without EGF (−EGF), and in the presence of 50 μM LY294002 (+LY) with or without EGF for 24 and 48 h. Cells were lysed and protein samples were evaluated for (A) apoptosis by immunoblotting for PARP cleavage products; (B) expression of Akt protein; (C) expression of Erk1/Erk2, (D) expression of Raf-1; (E) expression of PI3-K 85 kDa. The results are representative of two independent assays.

sence of the EGF for 48 h (Fig. 5E). The absence of Raf-1 protein downregulation further implicates the involvement of PI3-K/Akt pathway.

Downregulation of Akt protein is specific only for apoptosis induced through prolonged inhibition of PI3-K activity

To determine if the downregulation of Akt protein is simply the result of ongoing apoptosis in Myc83 cells, regardless of which of the survival pathways has been blocked, we treated Myc83 cells with 1 μM of PD153035, a specific inhibitor of the EGFR; with 10 μM of U0126 (a MEK inhibitor), and with 50 μM of LY294002 (PI3-K/Akt inhibitor) for 48 h. The samples were then evaluated for apoptosis by Hoechst staining and by PARP cleavage, as well as by immunoblotting for Akt protein expression. In the presence of EGF, only 2-3% of Myc83 cells underwent apoptosis. However, when EGF was removed, the number of apoptotic cells increased to 9% (Fig. 6A). When cells were treated with PD153035, in the presence of EGF, a total of 42% of the cells underwent apoptosis (Fig. 6A), while after removal of the EGF, 49% of the cells were apoptotic. In the presence of EGF and U0126, a total of 15% of the cells underwent apoptosis (Fig. 6A), while after removal of EGF almost 23% of the cells were apoptotic (Fig. 6A). A total of 22% of the Myc83 cells treated with EGF and LY294002 underwent apoptosis after 48 h; following EGF removal, treatment with LY294002 caused 33% of the cells to apoptose (Fig. 6A). In addition, we treated a separate set of Myc83 cells with the same inhibitors, under the same conditions for 48 h, and then lysed the cells for PARP protein analysis by immunoblotting. As expected, the results of PARP cleavage (Fig. 6B) confirmed the apoptosis results from the Hoechst staining (Fig. 6A). Interestingly, only those cells treated with an inhibitor of PI3-K (LY294002) for 48 h exhibited a significant decrease (2-fold) in the expression of the Akt (Fig. 6C). However, regardless of the apoptosis occurring in cells treated with other pharmacological inhibitors (PD153035 and U0126), the expression of Akt protein was not affected (Fig. 6C). When the Myc83 cells were treated with 60 ng/ml EGF, apoptosis induced by PD153035 treatment was inhibited up to ~70% as measured by Hoechst staining (data not shown). This shows the ability of EGF to rescue apoptosis of Myc83 cells.

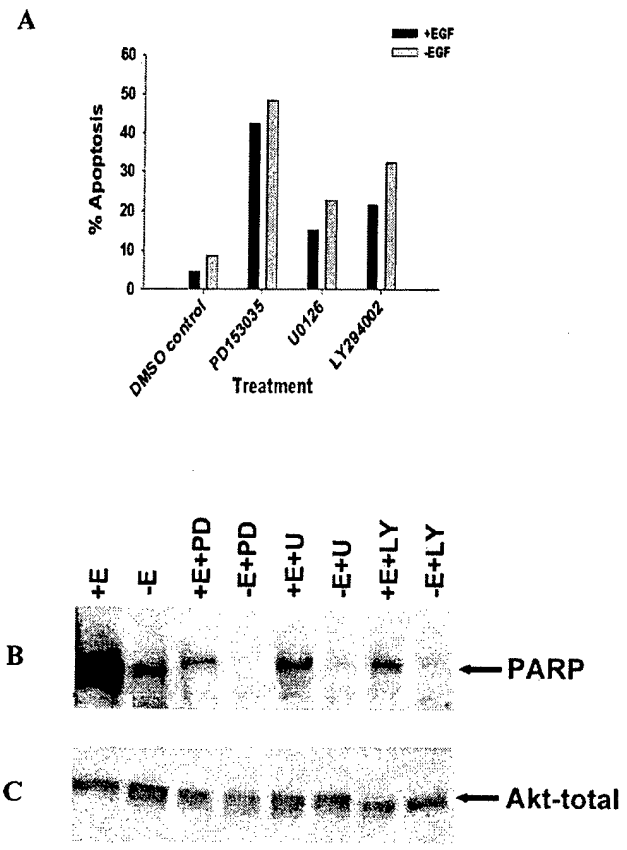
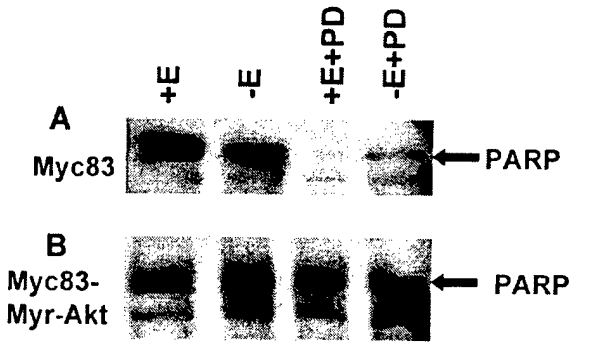


Fig. 6. Downregulation of Akt protein is specific for apoptosis induced by the inhibition of PI3-K activity. Myc83 cells were grown in IMEM medium with or without 10 ng/ml EGF, and in combination with 1 μM PD153035, 10 μM U0126, or 50 μM LY294002 for 48 h. Apoptosis was evaluated by (A) Hoechst staining (a total of 500 cells were counted) and (B) immunoblotting of PARP cleavage. (C) Akt expression was evaluated by immunoblotting using antibody-recognizing Akt independent of phosphorylation status. The results are representative of two independent assays.

Constitutively activated Akt protects MMECs overexpressing c-Myc from apoptosis

To further confirm the potential importance of Akt to deliver a survival signal downstream of EGFR, we retrovirally transduced constitutively activated Akt (Myr-Akt) into the Myc83 cells. In contrast to the parental Myc83 cells, previously shown to undergo apoptosis within 48 h of the removal of EGF and serum, Myc83-Myr-Akt cells were highly resistant to apoptosis on removal of EGF and serum from the growth medium, for up to 5 days (data not shown). The apoptotic response was evaluated by measuring PARP cleavage (Fig. 7A). PARP cleavage was detected in apoptotic pBabe-Myc83 cells treated with PD153035 for 48 h, while in Myc83-Myr-Akt cells, treated under the same conditions for 48 h, cells were protected from c-Myc-mediated apoptosis regardless of the presence of EGF or PD153035 (Fig. 7B).

Furthermore, the apoptotic profile of Myc83-Myr-Akt cells was additionally evaluated by Hoechst staining following treatment with 1 μM PD153035, 10 μM U0126, and 50 μM LY294001 for 48 h. Again, constitutively active Akt in Myc83-Myr-Akt cells almost completely protected the cells from apoptosis, resulting from the inhibition of EGFR kinase activity by PD153035 (Fig. 7C). After treatment with PD153035, in the presence of EGF, only 8% of the Myc83-Myr-Akt cells underwent apoptosis, in comparison to 42.4%



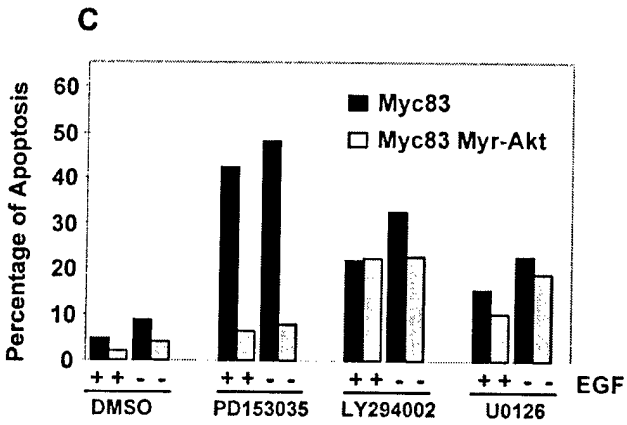


Fig. 7. Constitutively activated Akt protects MMECs overexpressing c-Myc from apoptosis. Myc83 (A) and Myc83-Myr-Akt (B) cells were grown in IMEM medium with or without EGF, and in combination with 1 μM PD153035 for 48 h. Apoptosis was analyzed by immunoblotting of PARP cleavage and Hoechst staining (C). The results are representative of two independent assays.

of Myc83 control cells (Fig. 7C). After the removal of EGF, in the presence of PD153035, a similar percentage of Myc83-Myr-Akt cells underwent apoptosis (7.4%), compared with 48.4% of control Myc83 cells in the same experiments. These data suggest that, despite the prolonged inhibition of EGFR kinase activity, the presence of constitutively activated Akt provides full protection from apoptosis in c-Myc-overexpressing MMECs.

Interestingly, constitutively activated Akt protected from apoptosis only 27% of the Myc83-Myr-Akt cells, in comparison to 22% of Myc83 in the presence of EGF plus the inhibitor of the PI3-K pathway (LY294002). Under the same conditions (in the presence of LY294002), when EGF was removed, only 29% of Myc83-Myr-Akt cells underwent apoptosis, compared with 32.5% of Myc83 cells. This result is not surprising, due to the fact that the myristolyation signal has been shown to decrease following prolonged inhibition of PI3-K activity in fibroblasts, leading to a decrease in Akt activity [24]. Also, because we detected proteolysis of endogenous Akt protein under the same conditions of prolonged inhibition of PI3-K activity for 48 h (Fig. 7C), Myr-Akt might be targeted to proteolysis as well. As expected, the constitutively activated Akt in Myc83-Myr-Akt cells did not provide significant protection from apoptosis induced by prolonged inhibition of the MEK/Erk pathway with U0126 (Fig. 7C).

Mechanism by which constitutively activated Akt inhibits apoptosis in c-Myc-overexpressing cells involves upregulation of Bcl-x_L

In fibroblasts and hematopietic cells, activated Akt has been shown to inhibit apoptosis, mostly through mechanisms involving inhibition of the pro-apoptotic Bad protein [8,12] caspase 9 [13] and inhibition of release of cytochrome c [24]. However, little information is available on the mechanism whereby Akt inhibits apoptosis in MMECs

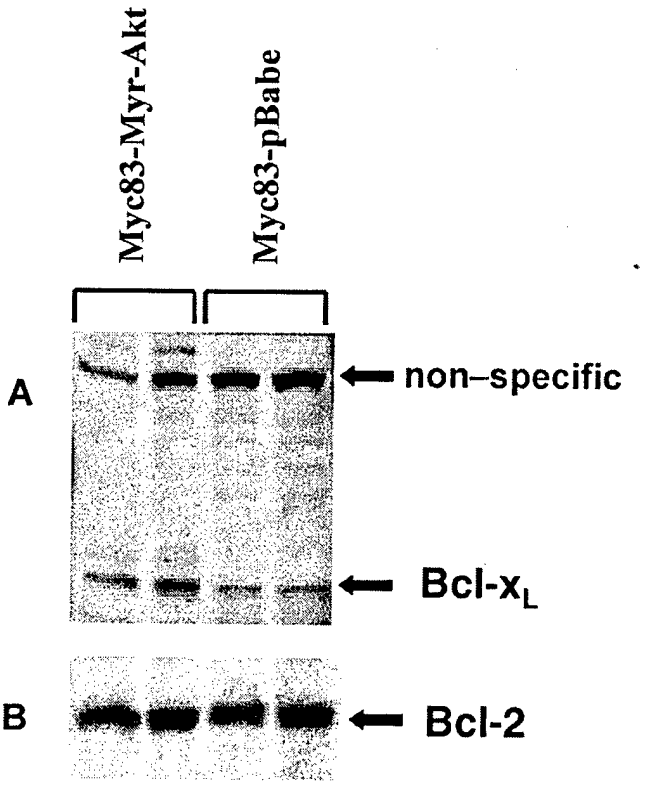


Fig. 8. Mechanism by which Akt protects MMECs from apoptosis involves the upregulation of Bcl-x_L. (A) Myc83 and Myc83-Myr-Akt were grown in IMEM medium containing 2.5% FCS, 10 ng/ml EGF (UBI), and 5 μ/ml insulin (Biofluids) Rockville, MD, USA for 48 h. Cells were lysed and protein samples analyzed by immunoblotting for the expression of Bcl-x_L protein. To increase the accuracy of the loading the same samples were loaded twice. (B) The same lysates were analyzed for Bcl-2 expression by immunoblotting. The results are representative of three independent assays.

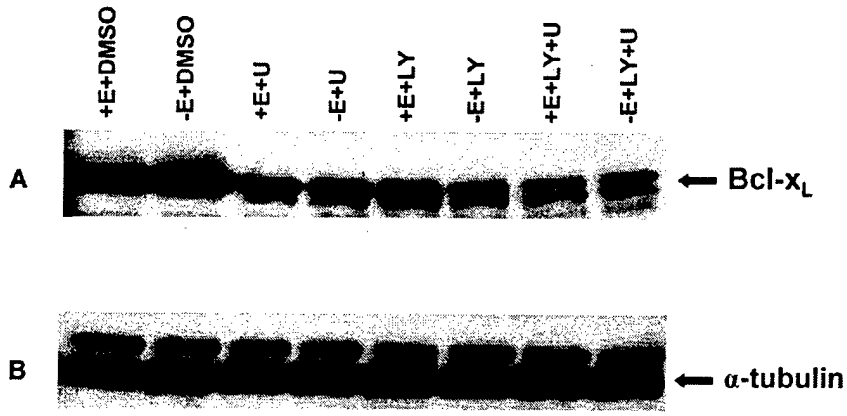


Fig. 9. Effects of pharmacological inhibitors of PI3-K/akt and MEK/Erk1/2 on Bcl-x_L protein expression in MMECs. (A) Effects of MEK inhibitor U0126 (10 μM) and PI3-K/Akt kinase inhibitor LY294002 (50 μM) on Bcl-x_L expression were measured by immunoblotting. Myc83 cells were treated with IMEM medium containing EGF and DMSO, and DMSO without EGF, and with pharmacological inhibitors with and without EGF for 48 h. Cells were lysed and protein samples were analyzed for expression of the Bcl-x_L protein. (B) The blots were reprobed with α-tubulin antibody to demonstrate equal loading. The results are representative of two independent assays.

and human breast cells and the mechanism by which Akt inhibits c-Myc-mediated apoptosis in general.

In our previous work we observed that one of the molecules upregulated by activation of EGFR in Myc83 cells is the prosurvival Bcl-x_L protein, at both the mRNA and protein levels [4]. Therefore, to explore if the mechanism whereby activated EGFR inhibits c-Myc-induced apoptosis involves Akt in our model, we analyzed the status of Bcl-x_L in parental Myc83, pBabe-Myc83, and Myc83-Myr-Akt cells grown in the presence of complete medium. Western blot analysis detected a significant, twofold increase (P = 0.005) in Bcl-x_L protein levels in Myc83-Myr-Akt cells, in comparison to Myc-83 cells transfected with vector only to pBabe-Myc83 cells (Fig. 8A).

In addition, we analyzed expression of the pro-survival Bcl-2 (Fig. 8B), 14-3-3 (data not shown), and other survival molecules, and no changes in expression were detected. Furthermore, we evaluated expression of the pro-apoptotic Bad protein, a known target of Akt. Although Bad expression levels did not differ between Myc83 and Myc-Myr-Akt cells (data not shown), it was difficult to determine the phosphorylation status of endogenous Bad in these cells by commercially available antibodies.

Effects of pharmacological inhibitors of PI3-k/Akt and Mek/Erk on Bcl-x_L protein expression

To test the role of both PI3-K/Akt and MEK/Erk1/2 pathways in the regulation of Bcl-x_L expression, we evaluated the effects of pharmacological inhibitors of these pathways on Bcl-x_L protein expression, as determined by immunoblotting. These experiments were done after 48 h of incubation with the respective compound. As shown in Fig. 9A, treatment with the MEK inhibitor U0126 in the presence (P = 0.016) and absence (P = 0.06) of EGF caused a twofold decrease in Bcl-x_L protein expression. Similarly, inhibition of the PI3-K/Akt pathway with LY294002 was associated with twofold downregulated Bcl-x_L protein expression, particularly when EGF had been removed from growth medium (P = 0.04) However, treatment with a combination of LY294002 and U0126 caused a highly significant threefold decrease in protein levels of Bcl-x_L in the presence of EGF (P = 0.003) and absence of EGF (P = 0.02). This indicates the importance of both pathways in regulation of Bcl-x_L expression. To test equal loading, blots were probed with antibody for α-tubulin (Fig. 9B).

Dominant-negative Akt and Erk1 effect the expression of Bcl-x_L

To obtain further independent evidence for the requirement of Akt and Erk signaling for expression of Bcl-x_L in Myc83, we transiently expressed DN-Akt and DN-Erk1. Similar to the PI3-K/Akt inhibitor LY294002, overexpressed DN-Akt induced Myc83 spontaneous apoptosis over a 48-h period (data not shown), and caused a significant ~40% reduction (P = 0.016) in the expression of Bcl-x_L protein (Figs. 10A and C). However, the reduction of Bcl-x_L protein expression in samples transfected with DN-Erk1 was only ~20% (P = 0.06) (Figs. 10A and C) in comparison to vector control. The effects of DN-Erk1 were less prominent in comparison to those of the specific MEK inhibitor U0123. To test for equal loading samples blots were reprobed with antibody for α-tubulin (Fig. 10B).

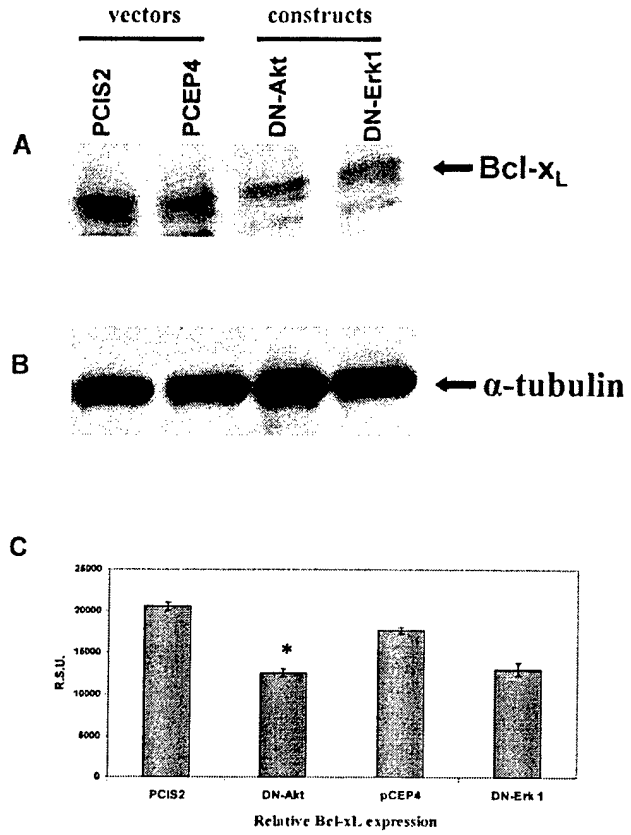


Fig. 10. Overexpression of DN-Akt and Erk1 in MMECs caused a reduction in Bcl-x_L protein. (A) Western immunoblotting analysis for expression of Bcl-x_L protein in Myc83 cells transiently transfected with vector controls (PCIS2 and pCEP4) and DN-Akt and DN-Erk1. (B) The equality of the loading was determined by reprobing blots with α-tubulin antibody. (C) R.S.U., relative scan units (with standard deviations indicated), are used to express the densitometrically measured Bcl-x_L signal to the α-tubulin in each sample. The results are representative of two independent assays. *, significantly different.

Discussion

Increased activation of EGFR [28–31] and dysregulated expression of c-Myc [1,2] are both commonly observed in human breast cancers. We previously described the dramatic interaction of these two tumor-associated aberrations in a bitransgenic model of human mammary cancer. Our studies showed that EGFR strongly suppressed c-Myc-mediated apoptosis by pro-survival signaling [3]. Signaling pathways linking EGFR to cellular survival in the context of inhibition of the pro-apoptotic state induced by c-Myc are not well defined in either mouse mammary or human breast cancer epithelial cells. We now provide evidence that EGF activates Akt/PKB and Erk1/2 in MMECs that overexpress c-Myc, and that both pathways inhibit c-Myc-mediated apoptosis, with Akt providing stronger survival signaling. These results may be specific for epithelial cells because in fibroblasts, EGF did not cause significant activation of Akt, nor was it able to protect cells from apoptosis [8].

Akt has been shown previously to inhibit apoptosis in fibroblasts, neuronal cells, hematopoietic cells, and some epithelial cells [8,9], but its role in apoptotic inhibition of mouse mammary and human breast cancer cells overexpressing c-Myc is unexplored. Recently, EGF has been reported to activate Akt in rat fetal hepatocytes [32] and in two human breast cancer cell lines, MCF-7 and T47D [33]. Our results are among the first demonstrating the importance of Akt activation in conveying survival signals downstream of EGFR in MMECs overexpressing c-Myc.

In the current study we show that constitutively activated Akt inhibits apoptosis, while upregulating protein levels of the pro-survival molecule Bcl-x_L. Furthermore, inhibition of PI3-K/Akt and MEK/Erk pathways by pharmacological inhibitors led to a significant, threefold decrease in Bcl-x_L protein expression, indicating the importance of both pathways in the regulation of Bcl-x_L protein. However, based on the results obtained with DN-Akt and DN-Erk1, it appears that Akt might have a more important role in the regulation of Bcl-x_L. This finding is consistent with our previous data, where we showed that EGF delivers survival signaling in these same c-Myc-overexpressing MMECs in association with upregulation of Bcl-x_L, at both the mRNA and protein levels [4]. Similarly, in mouse hepatocytes, EGF exerts its anti-apoptotic action partially through upregulation of Bcl-x_L [34], and EGF receptor signaling inhibits keratinocyte apoptosis through increased expression of endogenous mRNA and protein levels of Bcl-x_L [35]. However, it is not clear which signaling molecules downstream of EGFR are involved in the upregulation of Bcl-x_L. Our results here are the first indication that Bcl-x_L could be upregulated by Akt in models of human breast cancer. Currently, we are in the process of further investigating this finding in an attempt to elucidate the mechanism by which Akt upregulates Bcl-x_L.

Based on our work it seems that, contrary to what has been found in fibroblasts, Akt can regulate Bcl-x_L expression in mouse mammary epithelial cells. Similarly, it has been reported that Akt promotes T-lymphocyte survival through enhanced expression of Bcl-x_L protein in vivo, without affecting its mRNA level [15]. In rat pheochromocytoma (PC12) cells, Akt upregulates Bcl-2 expression through the c-AMP response element-binding protein [16], but any effect on Bcl-x_L has not been addressed. Finally, recent reports indicate that PI3-K activity can induce Bcl-x_L expression, at both the mRNA and protein levels in Baf-3 cells, but this study does not implicate Akt directly [36].

Bcl-x_L has been shown to insert into the mitochondrial membrane and form ion channels [37], directly controlling

mitochondrial cytochrome c release [38]. In both prior studies, the mitochondrial membrane potential was affected, and apoptosis was inhibited [37,38]. In some studies, Bcl-x_L has been shown to interact with caspase 9 and with apoptotic protease-activating factor (APAF-1), resulting in apoptotic inhibition [39]. It has been shown in hematopoetic cells that Akt and Bcl-x_L promote interleukin-3-independent survival through distinct effects on mitochondrial physiology [40]; however, it is not clear if this is the case in epithelial cells, including mouse mammary and breast cancer cells. The mechanism by which Akt, Erk, and upregulated Bcl-x_L contribute to apoptosis inhibition in our model is currently under investigation. There is a possibility, currently under exploration, that a twofold upregulation of Bcl-x_L protein could play a role in inhibition of cytochrome c release in c-Myc-overexpressing MMECs. In fibroblasts, the release of cytochrome c was reported to be involved in c-Myc-induced apoptosis [41].

Although the role of Akt in human breast cancer and MMECs is not completely understood, the role of Erk/MAPK in transformation of mouse mammary cells and in cancerous human breast tissue is firmly established in the literature [42]. Here we found that EGF activates Erk and Akt within a similar time frame. In addition, we have found that, although potentially less potent than PI3-K/Akt, the MAPK/Erk pathway also delivers a survival signal in Myc83 cells. Supporting this finding, Erk has been reported to deliver a survival signal in human chondrocytes [43] and in human neutrophils [44], and it was shown to cooperate with Akt in delivering survival signal to MCDK cells [26]. Recently, it has been also shown that one of the ways in which the MEK/Erk pathways induce survival in keratinocytes involves expression of Bcl-x_L [28]. Based on our results here, we predict the interaction of Akt and Erk in delivering survival through common targets in MMECs. One such target for both pathways could be Bcl-x_L.

In our study we have also noted a PI3-K-dependent downregulation of both Akt and Erk protein levels, resulting from the prolonged (24–48 h) inhibition of PI3-K activity. Although it was previously reported that Akt is targeted by caspases during Fas-induced apoptosis of human Jurkat cells and UV-irradiated U937 cells [27], it seems that caspases are not targeting Akt in Myc83 cells. Presently, it is not clear what are the reasons for these differences, but it could be potentially explained by the species differences. It seems that in Myc83 cells, the mechanism responsible for cleavage of both Akt and Erk, may not involve caspases. When we blocked the activity of EGFR and Erk/MAPK kinase, we could not detect Akt or Erk proteolysis (Fig. 6C), despite the induction of apoptosis (Figs. 6A and B). Thus, the proteolysis of Akt and Erk may be dependent on selective inhibition of PI3-K. In addition, it appears that PI3-K regulates the expression levels of Erk1 as well. The mechanism(s) of these PI3-K effects is not known at present. Our findings on Erk downregulation are consistent with a recent report, which showed that the inhibitors of PI3-K activity could block the Erk/MAPK kinase signaling pathway [45]. Also, PI3-K has been shown to control the activity of Erk/MAPK through Raf, a molecule regulating Erk function [46].

For the first time, we have demonstrated that activated Akt and Erk could be responsible for the EGFR-regulated overexpression of Bcl-x_L in models of human breast cancer. These findings may be of importance considering the unexplored role of both Akt and the Bcl-x_L in mouse mammary tumors and human breast cancer. Recently, Akt and Bcl-x_L have both been viewed as potential therapeutic targets in several human cancers and our findings could be a significant contribution. Furthermore, overexpressed Bcl-x_L protein is being considered as a prognostic marker in a few studies of breast carcinomas where its overexpression has been associated with advanced-stage disease.

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